IAI Accepts published online ahead of print

153 *gingivalis* ATCC 33277 (10^8 cells/ml) for 30 min in the presence or absence of envelopes
154 isolated from wild-type or mutant *P. gingivalis*. TNF-α-pretreated HUVECs were
155 incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml) for 30 min in the presence or
156 absence of purified FimA fimbriae and Pgm6/7.
157 **Measurement of VWF and nitric oxide.** HUVECs (3.5 x 10^5 cells/ml) were
158 seeded into 12-well plates and grown overnight. Then the cells were stimulated with 10
159 ng/ml of TNF-α for 3 h. *P. gingivalis* cells were inoculated into cultures at an MOI of 100
160 and the cultures were incubated for 30 min and 1 h. The culture media were then
161 collected and centrifuged at 13,000 rpm for removing bacterial cells. Concentrations of
162 VWF in the supernatants were measured using ELISA according to the manufacturer's
163 instructions (VWF ELISA kit, American Diagnostic Inc, Stanford, CT). The
164 concentration of NO_2^-/NO_3^- was also measured by 2,3-diaminonapthalene (DAN) assay
165 (24) .
166 **Preparation of *P. gingivalis* envelope.** Separation of whole envelopes and the
167 outer membrane from *P. gingivalis* strains was performed essentially as described previously
168 (30). Briefly, bacterial cells were washed with PBS (pH 7.5) containing 0.15 M NaCl and
169 then resuspended with PBS (pH 7.5) containing 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl
170 ketone, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin. The cells were
171 disrupted by sonication, and the remaining undisrupted bacterial cells were removed by
172 centrifugation at 1,000 x *g* for 10 min. The envelope was collected as a pellet by
173 centrifugation at 100,000 x *g* for 60 min at 4°C. The pellet was washed once by resuspension
174 in PBS and recentrifuged. The final pellet was suspended in PBS.
175 **Purification of FimA.** Major fimbriae from *P. gingivalis* ATCC 33277 was
176 purified as described previously (52). The purity was ascertained by scanning of the
177 stained sodium dodecyl sulfate (SDS)-polyacrylamide gel.

178 **Purification of Pgm6/7 complex.** Functional Pgm6/7 complex was purified by
179 two methods. First, we purified it electrophoretically from bacterial envelopes as
180 previously reported (32). Briefly, an envelope fraction of *P. gingivalis* was subjected to
181 SDS-PAGE under non-reducing conditions. A 120-kDa protein band, corresponding to
182 Pgm6/7 heterotrimer, was excised, and then the complex was extracted electrically from a
183 piece of gel. We used these samples for experiments of Figure 3E and supplemental
184 data#3B. Second, we constructed C-terminally hexahistidine-tagged Pgm6 and purified
185 Pgm6/7 complex by using a nickel affinity column from a *P. gingivalis* mutant. Briefly, we
186 inserted a DNA fragment consisting of *pgm6 orf* associated with the DNA sequence encoding
187 Gly-Ser-Ser-hexahistidine into the vector pT-COW (13) bearing a powerful promoter of the
188 350-bp upper region of *ragA* (31). The constructed plasmid was introduced into a
189 *pgm6*-deletion mutant of *P. gingivalis* (32). The cell lysate was applied to a nickel affinity
190 column and the bound proteins were eluted. Although a hexahistidine tag was associated
191 with Pgm6 alone, Pgm6/7 complex was obtained. We used these samples for experiments
192 of Figure 3F, 3G, and supplemental data #3C.

193

194

IAI Accepts published online ahead of print

195 RESULTS

196 **TNF-α augments adherence of *P. gingivalis* to endothelial cells through**
197 **inducing expression of E-selectin.** We first examined induction of E-selectin expression
198 by TNF-α using ELISA and Western blotting in HUVEC cultures. TNF-α induced a
199 time-dependent expression of E-selectin in HUVECs (supplemental data #1, #2). E-selectin
200 expression was maximal at 3 h after TNF-α addition. No basal expression of E-selectin was
201 found. To determine whether E-selectin expression in endothelial cells is involved in
202 adhesion of *P. gingivalis* to the cells, we incubated HUVECs with TNF-α (10 ng/ml) for 0.5-3
203 h, and then *P. gingivalis* ATCC 33277 cells (10^8 cells/ml/well) were added to the culture
204 medium for 0.5-3 h. Cells were then washed and attachment of *P. gingivalis* to the cells was
205 observed by fluorescent microscopy. Attachment of *P. gingivalis* to HUVECs increased
206 time-dependently without pretreatment of TNF-α (Figures 1A, B). Pretreatment with 10
207 ng/ml of TNF-α significantly enhanced the level of attachment in HUVEC cultures.
208 To clarify the role of E-selectin in *P. gingivalis* adherence to HUVECs, we examined the
209 effect of anti-E-selectin antibodies on *P. gingivalis a*dherence to HUVECs. HUVECs were
210 pretreated with TNF-α and were then incubated with *P. gingivalis* for 30 min in the presence
211 of antibodies for E-selectin or control IgG. An antibody to E-selectin inhibited *P. gingivalis*
212 adherence to TNF-α-pretreated HUVECs (Figure 2A).

213 E-selectin mediates the rolling of leukocytes on activated endothelial cells through
214 binding of the carbohydrate antigen sialyl Lewis X (37). Therefore, we examined the effect
215 of sialyl Lewis X on interactions between *P. gingivalis* and endothelial cells. Sialyl Lewis
216 X inhibited TNF-α-induced *P. gingivalis* adherence to HUVECs at a concentration of 0.1
217 μg/ml (Figure 2B). To assess the effect of E-selectin over-expression on the up-regulation
218 of *P. gingivalis* adherence to endothelial cells, we transfected a E-selectin-inserted plasmid
219 into HUVECs. Expression of E-selectin was confirmed by Western blotting 24 h after

220 transfection (Figure 2C). Adherence of *P. gingivalis* significantly increased in
221 E-selectin-transfected HEK 293 cells (Figure 2D). These results suggest that
222 TNF-α augments *P. gingivalis* adherence to HUVECs through inducing expression of
223 E-selectin.
224 ***P. gingivalis*** **interacts with TNF-α-stimulated endothelial cells via its ~~outer~~**
225 **~~membrane protein~~ Pgm6/7.** The initial adherence of *P. gingivalis* to host cells is mediated
226 by multiple adhesins including FimA and HagB (44) (45). To determine whether an
227 interaction between major fimbriae occurs with E-selectin, we examined adherence to
228 endothelial cells of *P. gingivalis* defective in FimA alone (ΔFimA). TNF-α increased the
229 adherence to endothelial cells of FimA-deficient *P. gingivalis* as well as wild-type *P.*
230 *gingivalis* and the degrees of adherence were similar (Figures 3A, B). We next examined
231 whether a major outer membrane protein of *P. gingivalis* that which is homologous to OmpA
232 protein in *Escherichia coli*, Pgm6/7, mediates *P. gingivalis* mediates adherence to HUVECs.
233 The Pgm6/7-deficient mutant (ΔPgm6/7) was incubated with TNF-α-pretreated HUVECs
234 and attachment of *P. gingivalis* to the cells was observed. TNF-α increased adherence of
235 wild-type *P. gingivalis* to endothelial cells but failed to increase adherence of ΔPgm6/7 *P.*
236 *gingivalis* to endothelial cells (Figure 3C). To clarify whether Pgm6/7 mediates *P.*
237 *gingivalis* adherence to HUVECs, we prepared envelopes from wild-type, ΔFimA, and
238 ΔPgm6/7 *P. gingivalis* and examined the effects on interaction between wild-type *P.*
239 *gingivalis* and HUVECs. Envelope peptides from wild-type *P. gingivalis* or ΔFimA *P.*
240 *gingivalis* suppressed adherence of *P. gingivalis* to TNF-α-pretreated HUVECs (Figure 3D).
241 However, envelope peptides from ΔPgm6/7 *P. gingivalis* did not affect *P. gingivalis*
242 adherence. In addition, the Pgm6/7 fraction from *P. gingivalis* ATCC 33277 suppressed
243 TNF-α-augmented *P. gingivalis* adherence, but the FimA fraction from the same strain did
244 not (Figure 3E). Furthermore, purified Pgm6/7 inhibited TNF-α activation of *P. gingivalis*

IAI Accepts published online ahead of print

245 adherence to HUVECs at a concentration as low as 0.25 ng/ml (Figure 3F, G). These results
246 suggest that the *P. gingivalis* peptide Pgm6/7 plays a role in the adherence of *P. gingivalis* to
247 endothelial cells.

248 ***P. gingivalis* interaction with endothelial cells via E-selectin induces**
249 **endothelial exocytosis and NO production.** Finally, to determine whether
250 E-selectin-mediated adherence of *P. gingivalis* activates endothelial cells and increses
251 vascular inflammation, we investigated induction of vWF and nitric oxide in TNF-α-
252 pretreated endothelial cells by stimulation with *P. gingivalis*. HUVECs were incubated with
253 TNF-α (10 ng/ml) for 3 h and then the cells were washed and incubated with *P. gingivalis* for
254 0-1 h. Then release of vWF into the media was measured by ELISA. *P. gingivalis* triggers
255 endothelial exocytosis, as measured by endothelial release of VWF. Release of vWF by
256 stimulation with *P. gingivalis* was also enhanced by pretreatment of HUVECs with TNF-α
257 (Figure 4). TNF-α pretreatment of HUVECs before *P. gingivalis* stimulation for 30 min
258 significantly increased NO_2^- release into the media (Figure 5). An anti-E-selectin antibody
259 ~~also~~ inhibited activation by *P. gingivalis* of NO release from TNF-α-pretreated HUVECs.
260 These results suggest that *P. gingivlis* interaction with endothelial cells via E-selectin
261 activates the endothelial cells and enhances proinflammatory responses of the cells to the
262 bacteria.

263 **DISCUSSION**

264 *P. gingivalis* adherence to and invasion of endothelial cells has been reported by
265 several investigators (46) (9) . However, this is the first report on the adhesion of activated
266 endothelial cells by *P. gingivalis*. HUVECs activated with TNF-α increased the adherence
267 of *P. gingivalis* through E-selectin expression, interacting with the OmpA-like proteins
268 Pgm6/7 in *P. gingivalis*.

269 One of the initial events in atherogenesis is the activation of endothelial cells,
270 which then express cell surface adhesion molecules such as endothelial leukocyte adhesion
271 molecule (E-selectin), vascular cell adhesion molecule (VCAM-1), and intercellular
272 adhesion molecule (ICAM-1) (10) (22) (8). These endothelial adhesion molecules in turn
273 facilitate the attachment of blood leukocytes to endothelial surfaces (34). In the present study,
274 we demonstrated that one of the periodontopathogens adhere to endothelial cells via
275 E-selectin.

276 *P. gingivalis* can invade many cell types, including human oral epithelial cells (33)
277 (51), human gingival fibroblasts or epithelial cells (3) (26), human coronary artery smooth
278 muscle cells, and human coronary artery endothelial (HCAE) cells (11). Adhesion of *P.*
279 *gingivalis* to host cells is multimodal (27) and involves a variety of cell surface and
280 extracellular components, including fimbriae, proteases, hemagglutinins, and
281 lipopolysaccharides (LPS) (8). Among the large array of virulence factors produced by *P.*
282 *gingivalis*, the major fimbria (FimA), as well as cysteine proteinases (gingipains), contribute
283 to the attachment to and invasion of many types of mammalian cells including oral epithelial
284 cells (4) and endothelial cells. *P. gingivalis* strains deficient in FimA fimbriae had
285 attenuated capacity to adhere to and invade epithelial cells and endothelial cells (33) (46)
286 (51) . Invasive *P. gingivalis* strains and their purified fimbriae activates expression of
287 cytokines and cell adhesion molecules in endothelial cells (46) . However, our data showed

IAI Accepts published online ahead of print

288 that Pgm6/7 rather than FimA is associated with *P. gingivalis* adherence to TNF-α-treated
289 endothelial cells. Although we do not know exact mechanisms, *P. gingivalis* cells adhere to
290 activated endothelial cells through their Pgm6/7 in a manner different from the
291 fimbriae-integrin interaction. TNF-α activates endothelial cells to express adhesion
292 molecules as well as proinflammatory cytokine and chemokine receptors and promotes
293 synthesis and release of a variety of inflammatory cytokines and chemokines to thereby
294 support recruitment of activated leukocytes to an inflammatory lesion (38). TNF-α promotes
295 the inflammatory cascade within the arterial wall during development of atherosclerosis (1).
296 In addition, *P. gingivalis* has been detected within atherosclerotic plaques from vascular
297 tissues (54) (25). Therefore, TNF-α may also augment adherence of *P. gingivalis* as well as
298 that of leukocytes in part through inducing E-selectin expression. Weibel–Palade
299 bodies (WPBs) are endothelial granules that store von Willebrand factor (VWF) and other
300 vascular modulators (50) (48). Endothelial cells secrete WPBs in response to vascular
301 injury, releasing VWF, which triggers platelet rolling. Endothelial exocytosis is one of the
302 earliest responses to vascular damage and plays a pivotal role in thrombosis
303 and inflammation (29). In this study we demonstrated that *P. gingivlis* interaction with
304 endothelial cells via E-selectin activates the endothelial cells and enhances endothelial
305 exocytosis (Figure 4) and may enhance atherogenesis and thrombosis (e.g., Buerger disease)
306 (7) (23).
307 Pgm6/7 in *P. gingivalis*, which shares a low homology with *E. coli*
308 OmpA, exists as a heterotrimer comprising Pgm6 and Pgm7 and plays a role in the outer
309 membrane integrity in this organism. OmpA in *E. coli* K1 has been reported to interact with
310 a glycoprotein (Ecgp) of human brain microvascular endothelial cells for
311 invasion. Therefore, *P. gingivalis* invasion into endothelial cells should be investigated in
312 the near future, especially as to whether Pgm6/7 is involved in the invasion. How does

313 Pgm6/7 bind to E-selectin? The adhesion activity of E-selectin is mediated primarily by the
314 binding of sialyl Lewis X on the leukocyte to the carbohydrate-binding domain. E-selectin
315 recognizes the carbohydrate structure of sLeX. Pgm6/7 is also a glycoprotein and therefore
316 it may bind to E-selectin through its carbohydrate side chain. However, we need additional
317 experiments for revealing the mechanism.

318 Collectively, in the present study, we clarified a new host-pathogen interaction: an
319 interaction between Pgm6/7, a major outer membrane protein of *P. gingivalis,* and E-selectin
320 of activated endothelial cells. This finding raises the possibility that chronic infection of the
321 vasculature by pathogens such as *P. gingivalis* could exacerbate systemic vascular diseases,
322 such as coronary heart disease, stroke, and diabetes mellitus.
323

IAI Accepts published online ahead of print

324 **ACKNOWLEDGMENTS**

325 This work was supported by Grants-in-Aid for Scientific Research 22390354 and 21659436

326 (to K.M.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

327

328

329 REFERENCES

330 1. **Aggarwal, B. B., and K. Natarajan.** 1996. Tumor necrosis factors: developments
331 during the last decade. Eur Cytokine Netw 7:93-124.
332 2. **Amar, S., N. Gokce, S. Morgan, M. Loukideli, T. E. Van Dyke, and J. A. Vita.**
333 2003. Periodontal disease is associated with brachial artery endothelial dysfunction
334 and systemic inflammation. Arterioscler Thromb Vasc Biol 23:1245-1249.
335 3. **Amornchat, C., S. Rassameemasmaung, W. Sripairojthikoon, and S. Swasdison.**
336 2003. Invasion of Porphyromonas gingivalis into human gingival fibroblasts in vitro.
337 J Int Acad Periodontol 5:98-105.
338 4. **Andrian, E., D. Grenier, and M. Rouabhia.** 2006. Porphyromonas
339 gingivalis-epithelial cell interactions in periodontitis. J Dent Res 85:392-403.
340 5. **Brown, L. J., R. C. Oliver, and H. Loe.** 1989. Periodontal diseases in the U.S. in
341 1981: prevalence, severity, extent, and role in tooth mortality. J Periodontol
342 60:363-370.
343 6. **Burt, B.** 2005. Position paper: epidemiology of periodontal diseases. J Periodontol
344 76:1406-1419.
345 7. **Chen, Z., M. Takahashi, T. Naruse, T. Nakajima, Y. W. Chen, Y. Inoue, I.**
346 **Ishikawa, T. Iwai, and A. Kimura.** 2007. Synergistic contribution of CD14 and
347 HLA loci in the susceptibility to Buerger disease. Hum Genet 122:367-372.
348 8. **Cutler, C. W., J. R. Kalmar, and C. A. Genco.** 1995. Pathogenic strategies of the
349 oral anaerobe, Porphyromonas gingivalis. Trends Microbiol 3:45-51.
350 9. **Deshpande, R. G., M. B. Khan, and C. A. Genco.** 1998. Invasion of aortic and
351 heart endothelial cells by Porphyromonas gingivalis. Infect Immun 66:5337-5343.
352 10. **Dong, Z. M., S. M. Chapman, A. A. Brown, P. S. Frenette, R. O. Hynes, and D. D.**
353 **Wagner.** 1998. The combined role of P- and E-selectins in atherosclerosis. J Clin

354 Invest 102:145-152.
355 11. **Dorn, B. R., W. A. Dunn, Jr., and A. Progulske-Fox.** 1999. Invasion of human
356 coronary artery cells by periodontal pathogens. Infect Immun 67:5792-5798.
357 12. **Dorn, B. R., W. A. Dunn, Jr., and A. Progulske-Fox.** 2001. Porphyromonas
358 gingivalis traffics to autophagosomes in human coronary artery endothelial cells.
359 Infect Immun 69:5698-5708.
360 13. **Gardner, R. G., J. B. Russell, D. B. Wilson, G. R. Wang, and N. B. Shoemaker.**
361 1996. Use of a modified Bacteroides-Prevotella shuttle vector to transfer a
362 reconstructed beta-1,4-D-endoglucanase gene into Bacteroides uniformis and
363 Prevotella ruminicola B(1)4. Appl Environ Microbiol 62:196-202.
364 14. **Griffen, A. L., M. R. Becker, S. R. Lyons, M. L. Moeschberger, and E. J. Leys.**
365 1998. Prevalence of Porphyromonas gingivalis and periodontal health status. J Clin
366 Microbiol 36:3239-3242.
367 15. **Haffajee, A. D., and S. S. Socransky.** 2005. Microbiology of periodontal diseases:
368 introduction. Periodontol 2000 38:9-12.
369 16. **Hansson, G. K.** 2005. Inflammation, atherosclerosis, and coronary artery disease. N
370 Engl J Med 352:1685-1695.
371 17. **Hasegawa, Y., J. Iwami, K. Sato, Y. Park, K. Nishikawa, T. Atsumi, K.**
372 **Moriguchi, Y. Murakami, R. J. Lamont, H. Nakamura, N. Ohno, and F.**
373 **Yoshimura.** 2009. Anchoring and length regulation of Porphyromonas gingivalis
374 Mfa1 fimbriae by the downstream gene product Mfa2. Microbiology 155:3333-3347.
375 18. **Hashizume, T., T. Kurita-Ochiai, and M. Yamamoto.** Porphyromonas gingivalis
376 stimulates monocyte adhesion to human umbilical vein endothelial cells. FEMS
377 Immunol Med Microbiol 62:57-65.
378 19. **Heimdahl, A., G. Hall, M. Hedberg, H. Sandberg, P. O. Soder, K. Tuner, and C.**

IAI Accepts published online ahead of print

379 **E. Nord.** 1990. Detection and quantitation by lysis-filtration of bacteremia after
380 different oral surgical procedures. J Clin Microbiol 28:2205-2209.
381 20. **Herzberg, M. C., and M. W. Weyer.** 1998. Dental plaque, platelets, and
382 cardiovascular diseases. Ann Periodontol 3:151-160.
383 21. **Hope, S. A., and I. T. Meredith.** 2003. Cellular adhesion molecules and
384 cardiovascular disease. Part I. Their expression and role in atherogenesis. Intern Med
385 J 33:380-386.
386 22. **Iiyama, K., L. Hajra, M. Iiyama, H. Li, M. DiChiara, B. D. Medoff, and M. I.
387 Cybulsky.** 1999. Patterns of vascular cell adhesion molecule-1 and intercellular
388 adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at
389 sites predisposed to lesion formation. Circ Res 85:199-207.
390 23. **Iwai, T.** 2009. Periodontal bacteremia and various vascular diseases. J Periodontal
391 Res 44:689-694.
392 24. **Kleinhenz, D. J., X. Fan, J. Rubin, and C. M. Hart.** 2003. Detection of endothelial
393 nitric oxide release with the 2,3-diaminonapthalene assay. Free Radic Biol Med
394 34:856-861.
395 25. **Kurihara, N., Y. Inoue, T. Iwai, M. Umeda, Y. Huang, and I. Ishikawa.** 2004.
396 Detection and localization of periodontopathic bacteria in abdominal aortic
397 aneurysms. Eur J Vasc Endovasc Surg 28:553-558.
398 26. **Lamont, R. J., A. Chan, C. M. Belton, K. T. Izutsu, D. Vasel, and A. Weinberg.**
399 1995. Porphyromonas gingivalis invasion of gingival epithelial cells. Infect Immun
400 63:3878-3885.
401 27. **Lamont, R. J., and H. F. Jenkinson.** 1998. Life below the gum line: pathogenic
402 mechanisms of Porphyromonas gingivalis. Microbiol Mol Biol Rev 62:1244-1263.
403 28. **Libby, P.** 2002. Inflammation in atherosclerosis. Nature 420:868-874.

IAI Accepts published online ahead of print

404 29. **Matsushita, K., C. N. Morrell, B. Cambien, S. X. Yang, M. Yamakuchi, C. Bao,**
405 **M. R. Hara, R. A. Quick, W. Cao, B. O'Rourke, J. M. Lowenstein, J. Pevsner, D.**
406 **D. Wagner, and C. J. Lowenstein.** 2003. Nitric oxide regulates exocytosis by
407 S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell 115:139-150.
408 30. **Murakami, Y., M. Imai, H. Nakamura, and F. Yoshimura.** 2002. Separation of the
409 outer membrane and identification of major outer membrane proteins from
410 Porphyromonas gingivalis. Eur J Oral Sci 110:157-162.
411 31. **Nagano, K., Y. Murakami, K. Nishikawa, J. Sakakibara, K. Shimozato, and F.**
412 **Yoshimura.** 2007. Characterization of RagA and RagB in Porphyromonas gingivalis:
413 study using gene-deletion mutants. J Med Microbiol 56:1536-1548.
414 32. **Nagano, K., E. K. Read, Y. Murakami, T. Masuda, T. Noguchi, and F.**
415 **Yoshimura.** 2005. Trimeric structure of major outer membrane proteins homologous
416 to OmpA in Porphyromonas gingivalis. J Bacteriol 187:902-911.
417 33. **Njoroge, T., R. J. Genco, H. T. Sojar, N. Hamada, and C. A. Genco.** 1997. A role
418 for fimbriae in Porphyromonas gingivalis invasion of oral epithelial cells. Infect
419 Immun 65:1980-1984.
420 34. **Osterud, B., and E. Bjorklid.** 2003. Role of monocytes in atherogenesis. Physiol
421 Rev 83:1069-1112.
422 35. **Prasadarao, N. V., P. K. Srivastava, R. S. Rudrabhatla, K. S. Kim, S. H. Huang,**
423 **and S. K. Sukumaran.** 2003. Cloning and expression of the Escherichia coli K1
424 outer membrane protein A receptor, a gp96 homologue. Infect Immun 71:1680-1688.
425 36. **Read, M. A., A. S. Neish, F. W. Luscinskas, V. J. Palombella, T. Maniatis, and T.**
426 **Collins.** 1995. The proteasome pathway is required for cytokine-induced
427 endothelial-leukocyte adhesion molecule expression. Immunity 2:493-506.
428 37. **Rosen, S. D., and C. R. Bertozzi.** 1994. The selectins and their ligands. Curr Opin

429 Cell Biol 6:663-673.

430 38. **Ross, R.** 1999. Atherosclerosis--an inflammatory disease. N Engl J Med
431 340:115-126.

432 39. **Rothlein, R., M. Czajkowski, M. M. O'Neill, S. D. Marlin, E. Mainolfi, and V. J.**
433 **Merluzzi.** 1988. Induction of intercellular adhesion molecule 1 on primary and
434 continuous cell lines by pro-inflammatory cytokines. Regulation by pharmacologic
435 agents and neutralizing antibodies. J Immunol 141:1665-1669.

436 40. **Sandros, J., P. Papapanou, and G. Dahlen.** 1993. Porphyromonas gingivalis
437 invades oral epithelial cells in vitro. J Periodontal Res 28:219-226.

438 41. **Sconyers, J. R., J. J. Crawford, and J. D. Moriarty.** 1973. Relationship of
439 bacteremia to toothbrushing in patients with periodontitis. J Am Dent Assoc
440 87:616-622.

441 42. **Slots, J.** 1979. Subgingival microflora and periodontal disease. J Clin Periodontol
442 6:351-382.

443 43. **Slots, J., and M. Ting.** 1999. Actinobacillus actinomycetemcomitans and
444 Porphyromonas gingivalis in human periodontal disease: occurrence and treatment.
445 Periodontol 2000 20:82-121.

446 44. **Sojar, H. T., Y. Han, N. Hamada, A. Sharma, and R. J. Genco.** 1999. Role of the
447 amino-terminal region of Porphyromonas gingivalis fimbriae in adherence to
448 epithelial cells. Infection and immunity 67:6173-6176.

449 45. **Song, H., M. Belanger, J. Whitlock, E. Kozarov, and A. Progulske-Fox.** 2005.
450 Hemagglutinin B is involved in the adherence of Porphyromonas gingivalis to human
451 coronary artery endothelial cells. Infection and immunity 73:7267-7273.

452 46. **Takahashi, Y., M. Davey, H. Yumoto, F. C. Gibson, 3rd, and C. A. Genco.** 2006.
453 Fimbria-dependent activation of pro-inflammatory molecules in Porphyromonas

IAI Accepts published online ahead of print

454 gingivalis infected human aortic endothelial cells. Cell Microbiol 8:738-757.
455 47. **Tedder, T. F., D. A. Steeber, A. Chen, and P. Engel.** 1995. The selectins: vascular
456 adhesion molecules. FASEB J 9:866-873.
457 48. **Wagner, D. D., S. Saffaripour, R. Bonfanti, J. E. Sadler, E. M. Cramer, B.**
458 **Chapman, and T. N. Mayadas.** 1991. Induction of specific storage organelles by
459 von Willebrand factor propolypeptide. Cell 64:403-413.
460 49. **Wang, G., C. W. Woo, F. L. Sung, Y. L. Siow, and K. O.** 2002. Increased monocyte
461 adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of
462 chemokine and adhesion molecules. Arterioscler Thromb Vasc Biol 22:1777-1783.
463 50. **Weibel, E. R., and G. E. Palade.** 1964. New Cytoplasmic Components in Arterial
464 Endothelia. J Cell Biol 23:101-112.
465 51. **Weinberg, A., C. M. Belton, Y. Park, and R. J. Lamont.** 1997. Role of fimbriae in
466 Porphyromonas gingivalis invasion of gingival epithelial cells. Infect Immun
467 65:313-316.
468 52. **Yoshimura, F., K. Takahashi, Y. Nodasaka, and T. Suzuki.** 1984. Purification and
469 characterization of a novel type of fimbriae from the oral anaerobe Bacteroides
470 gingivalis. J Bacteriol 160:949-957.
471 53. **Yoshizaki, K., H. Wakita, K. Takeda, and K. Takahashi.** 2008. Conditional
472 expression of microRNA against E-selectin inhibits leukocyte-endothelial adhesive
473 interaction under inflammatory condition. Biochem Biophys Res Commun
474 371:747-751.
475 54. **Zaremba, M., R. Gorska, P. Suwalski, and J. Kowalski.** 2007. Evaluation of the
476 incidence of periodontitis-associated bacteria in the atherosclerotic plaque of
477 coronary blood vessels. J Periodontol 78:322-327.
478

479

480

IAI Accepts published online ahead of print

481 **FIGURE LEGENDS**

482 **Figure 1. Adherence of *P. gingivalis* to HUVECs was enhanced by stimulation with**
483 **TNF-α.** (A) HUVECs were incubated with TNF-α (10 ng/ml) for 0.5-3 h. Then *P.*
484 *gingivalis* ATCC 33277 cells (10^8 cells/ml/well) were added to the culture medium for 0.5-3
485 h. Cells were then washed and attachment of *P.gingivalis* to the cells was observed by
486 fluorescent microscopy. *P. gingivalis* was stained with FITC (green), and actin of endothelial
487 cells was visualized with TRITC (red). Scale bar is 10 μm. (B) HUVECs were incubated
488 with TNF-α (10 ng/ml) for 0.5-3 h. Then *P. gingivalis* ATCC 33277 cells (10^8 cells/ml/well)
489 were added to the culture medium for 0.5-3 h. Cells were then washed and attachment of *P.*
490 *gingivalis* to the cells was observed by fluorescent microscopy. The attachment levels were
491 expressed as number of *P. gingivalis* cells per 60430 mm^2 (n = 3, means ± SD; $*P < 0.01$ vs
492 no TNF-α).
493

494 **Figure 2. Adherence of *P. gingivalis* to TNF-α-activated endothelial cells was mediated**
495 **by E-selectin.** (A) Inhibitory effect of anti-E-selectin antibodies. HUVECs were
496 incubated with TNF-α (10 ng/ml) for 3 h. Cells were then washed and incubated with *P.*
497 *gingivalis* ATCC 33277 (10^8 cells/ml/well) for 30 min in the presence of an antibody for
498 E-selectin or control IgG. Other procedures are described in the legend to Fig. 1B. (n = 3,
499 means ± SD; $*P < 0.01$ vs no TNF-α, $†P < 0.01$ vs. no Anti-E-selectin Abs). (B) Inhibitory
500 effect of sialyl Lewis X. HUVECs were incubated with TNF-α (10 ng/ml) for 30 min.
501 Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml/well) for
502 3 h in the presence of purified sialyl Lewis X (0-10 ng/ml). Other procedures are described in
503 the legend to Fig. 1B. (n = 3, means ± SD; $*P < 0.01$ vs. no TNF-α, $†P < 0.01$ vs. no sialyl
504 Lewis X). (C) Adherence of *P. gingivalis* was augmented in HEK293 cells transfected with
505 an expression with vector for E-selectin. *P. gingivalis* ATCC 33277 (10^8 cells/ml/well) was

506 incubated with 293 cells transfected with a human E-selectin-inserted vector for 3 h. Other
507 procedures are described in the legend to Fig. 1A. Scale bar is 10 μm. (D) Adherence of *P.*
508 *gingivalis* was augmented in 293 cells transfected with an expression vector for E-selectin. *P.*
509 *gingivalis* ATCC 33277 (10^8 cells/ml/well) was incubated with 293 cells transfected with a
510 human E-selectin-inserted vector for 30 min. Other procedures are described in the legend
511 to Fig. 1B. (n = 3, means ± SD; **P* < 0.01 vs. control).
512

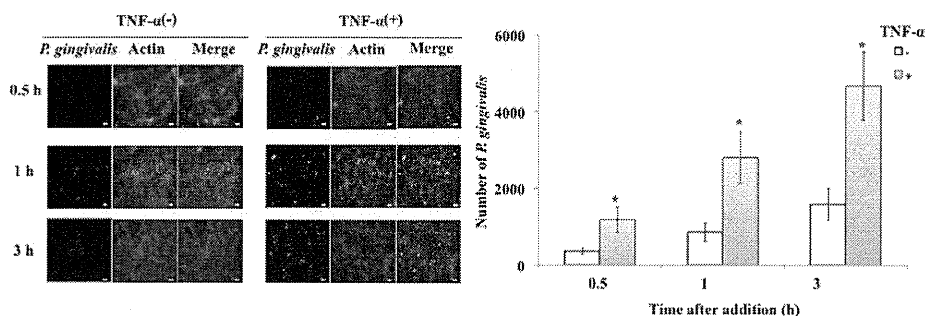
513 **Figure 3. Pgm6/7 in *P. gingivalis* mediated the interaction with activated endothelial**
514 **cells.** (A) *P. gingivalis* ATCC 33277 (wild type), FimA-deficient mutant (ΔFimA), and –
515 Pgm6/7-deficient mutant (ΔPgm6/7) (10^8 cells/ml/well) were incubated with
516 TNF-α-pretreated HUVECs for 3 h, respectively. Other procedures are described in the
517 legend to Fig. 1A. Scale bar is 10 μm. (B) *P. gingivalis* ATCC 33277 (wild type) and
518 FimA-deficient mutant (ΔFimA) (10^8 cells/ml/well) were incubated with TNF-α-pretreated
519 HUVECs for 30 min, respectively. Other procedures are described in the legend to Fig. 1A.
520 Scale bar is 10 μm. (C) *P. gingivalis* ATCC 33277 (wild type) and Pgm6/7-deficient mutant
521 (Pgm6/7) (10^8 cells/ml/well) were incubated with TNF-α-pretreated HUVECs for 30 min,
522 respectively. Other procedures are described in the legend to Fig. 1B. (n = 3, means ± SD;
523 **P* < 0.01 vs. no TNF-α). (D) Inhibitory effects of *P. gingivalis* envelopes on
524 TNF-α-induced adhesion of *P. gingivalis* to HUVECs. HUVECs were incubated with
525 TNF-α (10 ng/ml) for 30 min. Cells were then washed and incubated with *P. gingivalis* ATCC
526 33277 (10^8 cells/ml/well) for 30 min in the presence or absence of envelopes isolated from
527 wild-type or mutant *P. gingivalis*. Other procedures are described in the legend to Fig. 1B.
528 (n = 3, means ± SD; **P* < 0.01 vs. no TNF-α, †*P* < 0.01 vs. control). (E) Effects of extracted
529 ~~OmpA-like protein~~ Pgm6/7 and FimA on TNF-α-induced adhesion of *P. gingivalis* to
530 HUVECs. HUVECs were incubated with TNF-α (10 ng/ml) for 30 min. Cells were then

IAI Accepts published online ahead of print

531 washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ ml/well) for 30 min in the
532 presence or absence of purified Pgm6/7 and FimA. Other procedures are described in the
533 legend to Fig. 1B. (n = 3, means ± SD; **P* < 0.01 vs. no TNF-α, †*P* < 0.01 vs. Pgm6/7
534 fraction). (F) Inhibitory effect of *P. gingivalis* Pgm6/7 on TNF-α (10 ng/ml)-induced
535 adhesion of *P. gingivalis* to HUVECs. HUVECs were incubated with TNF-α (10 ng/ml) for
536 30 min. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/
537 ml/well) for 30 min in the presence or absence of purified Pgm6/7. Other procedures are
538 described in the legend to Fig. 1B. (n = 3, means ± SD; **P* < 0.01 vs. no TNF-α, †*P* < 0.01
539 vs. Pgm6/7 0 ng/ml). (G) Inhibitory effect of *P. gingivalis* Pgm6/7 on TNF-α-induced
540 adhesion of *P. gingivalis* to HUVECs. HUVECs were incubated with TNF-α (10 ng/ml) for
541 30 min. Cells were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8
542 cells/ml/well) for 30 min in the presence or absence of purified Pgm6/7. Other procedures
543 are described in the legend to Fig. 1A. Scale bar is 10 μm.
544
545 **Figure 4. Endothelial vWF exocytosis to *P. gingivalis* were augmented by pretreatment**
546 **with TNF-α.** HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells were then
547 washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells/ml/well) for 0-1 h. Then
548 the release of vWF into media was measured by ELISA. (n = 3, means ± SD)
549
550 **Figure 5. *P. gingivalis*-induced nitric oxide release from activated endothelial cells was**
551 **mediated by E-selectin.** HUVECs were incubated with TNF-α (10 ng/ml) for 3 h. Cells
552 were then washed and incubated with *P. gingivalis* ATCC 33277 (10^8 cells ml^{-1}/well) for 30
553 min in the presence or absence of an antibody for E-selectin. Then the release of nitric oxide
554 into media was measured by DAN assay (n = 3, means ± SD; **P* < 0.01 vs no TNF-α).
555

556

557 **A** **B**



558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587 **Komatsu *et al.* Fig. 1.**
588